real-time PCR. The phosphorylation of ERK was measured by ELISA. MKP-1 expression was measured by real-time PCR and western blotting.
Results: IL-6+sIL-6R, IL-1β and TNF-α induced MMP-3 and ADAMTS-4 mRNA in human articular chondrocytes in a concentration-dependent manner. Moreover, all three cytokines promoted the phosphorylation of ERK, whereas the total amount of ERK did not change. Both HA and ERK inhibitor completely inhibited MMP-3 and ADAMTS-4 mRNA induction by IL-6+sIL-6R or IL-1β, but both did not inhibit MMP-3 and ADAMTS-4 mRNA induction by TNF-α. Both HA and ERK inhibitor completely suppressed the phosphorylation of ERK induced by all cytokines in human articular chondrocytes.
To further analyze the inhibitory action of HA on ERK signal pathways induced by IL-6+sIL-6R and IL-1β, we tested whether HA could induce MKP-1, a negative regulator of ERK. HA treatment induced the expression of MKP-1 mRNA and its protein in human articular chondrocytes.
To determine the involvement of MKP-1 in HA-induced suppression of MMP-3 and ADAMTS-4 expressions induced by IL-6+sIL-6R and IL-1β, we examined whether MKP-1 inhibitor could block the effect of HA. MKP-1 inhibitor clearly reversed the suppressive effect of HA on MMP-3 and ADAMTS-4 expressions induced by IL-6+sIL-6R and IL-1β.
Conclusions: Our study clearly demonstrates that ERK involves in the induction of MMP-3 and ADAMTS-4 by IL-6+sIL-6R and IL-1β, but not by TNF-α. HA suppresses the induction of MMP-3 and ADAMTS-4 via the induction of MKP-1, a negative regulator of ERK.

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RECOMBINANT HUMAN FIBROBLAST GROWTH FACTOR-18 (RHFG18) PROMOTES BOVINE ARTICULAR CHONDROCYTE PROLIFERATION AND CARTILAGE MATRIX PRODUCTION *IN VITRO*.

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Purpose: An important goal in the treatment of patients with osteoarthritis and cartilage injury is to restore the articular surface of degraded or injured cartilage. Regeneration could be achieved by anabolic compounds stimulating extracellular matrix (ECM) molecule production and recellularization, thereby compensating for the matrix and cell loss occurring in OA cartilage. The present work sought to evaluate the ability of recombinant human fibroblast growth factor-18 (rhFGF18) to exhibit such properties in various *in vitro* and *ex vivo* systems.
Methods: Primary bovine articular chondrocytes were tested for expression of fibroblast growth factor receptor 3 (FGFR3, the main receptor for rhFGF18 signaling in cartilage) at the mRNA and protein level by quantitative polymerase chain reaction (qPCR) and Western Blot, respectively. A dose-response curve (0.1-10 000 ng/mL rhFGF18) was realized in monolayer to determine the best dosage. Next, 3D cultures with 10 and 100 ng/mL rhFGF18 were performed with rhFGF18 applied for 4 weeks (4w), or for 1 week followed by 3 weeks without rhFGF18 (1w). Cell proliferation was evaluated in monolayer using a cell counter. ECM production and the chondrocytic phenotype were investigated for both monolayer and 3D cultures by qPCR for collagen I, II, X, aggrecan and Sox9. Glycosaminoglycan (GAG) content of the 3D constructs was also evaluated with the dimethylmethylene blue assay after proteinase K digestion. Finally, the impact of rhFGF18 (100 ng/mL) on proliferation in bovine cartilage explants was assessed. For this purpose, cell counting using automated image analysis was done after 41 days of culture with/without rhFGF18. In a second trial, cell proliferation was quantified using proliferating cell nuclear antigen (PCNA) staining in histological sections in bovine cartilage explants after 0, 2, 4, 6 weeks' rhFGF18 administration.
Results: At both the protein and mRNA levels, primary bovine articular chondrocytes expressed FGFR3. In monolayer cell culture they responded to rhFGF18 with a dose-dependent increase in proliferation (1.9 fold at 10 ng/mL) and a dose-dependent decrease of collagen type I expression (64 fold at 100 ng/mL). In 3D culture (see Figure), short exposure to rhFGF18 100 ng/mL (1w) triggered GAG production (1.5 fold) and collagen type II expression (3.2 fold), while collagen type I expression was decreased (2.3 fold). Chondrocyte proliferation was also stimulated in bovine cartilage explants by 100 ng/mL rhFGF18, demonstrating its ability to reach the cells in cartilage. The maximum effect of rhFGF18 on explants was observed after 4 weeks in culture (11.25% PCNA positive cells with rhFGF18 treatment compared to 4.88% PCNA positive cells without rhFGF18).

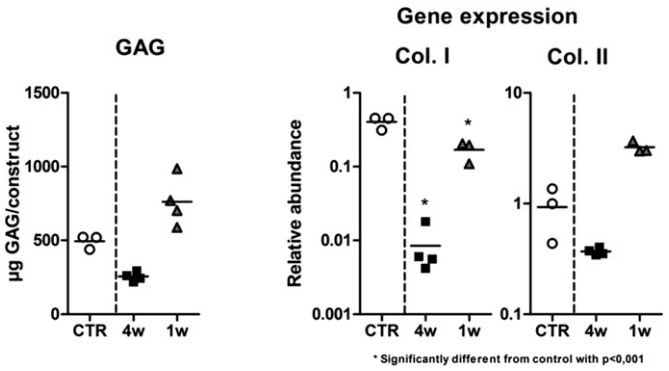

Figure: GAG content and collagen (Col.) types I and II gene expression in 3D constructs cultured in absence (CTR, control) or presence of rhFGF18 (100 ng/mL) over a 4-week culture period (4w) or for the first week (1w) only.
Conclusions: Taken together these results suggest that rhFGF18 can efficiently penetrate cartilage, and stimulate chondrocyte proliferation and cartilage ECM production. This is consistent with previous *in vitro* studies and further strengthens the rationale for the current clinical investigation of rhFGF18 as an anabolic disease modifying osteoarthritis drug (DMOAD).

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A UNIQUE TOOL TO SELECTIVELY DETECT THE CHONDROGENIC IIB FORM OF HUMAN PROCOLLAGEN II PROTEIN.

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Purpose: Type II collagen, the major fibrillar collagen of cartilage, is synthesized in precursor forms (procollagens) containing NH2- and COOH-terminal propeptides. Three splice variants are thought to be translated to produce procollagen II isoforms (IIA/IID and IIB) that differ in their aminopropeptide parts. The IIA and IID are transient embryonic isoforms which include an additional cysteine rich domain implicated in the regulation of TGF-β superfamily signalling. The IIA and IID transcripts are co-expressed during chondrogenesis then decline and the IIB isoform is the only one expressed and synthesized in mature fully differentiated chondrocytes. Besides, procollagen IIA can be re-expressed by dedifferentiating chondrocytes and in osteoarthritic cartilage. Therefore, it is an important point to determine which isoform(s) is (are) synthesized in vivo in normal and pathological situations and in vitro, to complete the phenotypic characterization of collagen II protein producing cells. Antibodies directed against the cysteine rich extradomain of procollagen IIA are already available and our goal in this study was to obtain and validate for the first time antibodies detecting only the chondrogenic IIB form of procollagen II.
Methods: We designed a triple peptide anti-peptide strategy to produce, to titer by ELISA and to affinity-purify rabbit polyclonal antibodies to human procollagen IIB. Human knee chondrocytes and the Saos-2 human osteoblastic cell line were cultured in various conditions to prepare cell extracts and media for the analysis of fibrillar procollagens synthesized and recognized by our antibodies to procollagen IIB by Western-blotting (WB).
Results: The antibodies to procollagen IIB (now referred to as anti-pNIIB52 antibodies) show a strong reactivity in WB with procollagen II and with pN-collagen IIB (the form lacking the COOH-terminal propeptide but still containing the NH2-terminal one) when compared to antibodies directed against the triple-helical part of type II collagen. These forms are the only bands revealed in the cell layer of chondrocytes cultured for 36 hours after

their isolation. The absence of cross-reaction with the IIA isoform was established by ELISA and WB. In addition, the Saos-2 cell line was chosen to test a possible labelling of other fibrillar procollagens, mainly type I, V and XI. In fact, this cell line is described to synthesize the (α1)I, (α2)I, (α1)V, (α2)V, (α1)XI and (α2)XI, but no (α3)XI chains. No signal was detected on WB of cellular extracts or conditioned media with anti-pNIIB52, whereas antibodies to the collagen I, V and XI triple-helical parts revealed indeed the presence of proforms of these collagens.

Conclusions: Anti-pNIIB52 antibodies allow a very sensitive and specific detection of the procollagen IIB, the protein synthesized by mature chondrocytes. No cross-reactions with the IIA or with the major fibrillar procollagens synthesized by cells with an osteoblastic phenotype were observed. These antibodies offer a new tool in basic as well as in translational research concerning cartilage differentiation, homeostasis and repair.

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INFLUENCE OF OXYGEN TENSION ON THE ANTI-INFLAMMATORY AND CHONDROPROTECTIVE EFFECTS OF HEME OXYGENASE-1 IN HEALTHY AND OSTEOARTHRITIC HUMAN CHONDROCYTES

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Purpose: Articular chondrocytes are adapted to live in conditions of low O_2. Such hypoxic conditions in the avascular cartilage play an important role in extracellular matrix synthesis and survival of chondrocytes. In previous work we have shown anti-inflammatory and chondroprotective effects of heme oxygenase-1 (HO-1) on osteoarthritic (OA) condrocytes in primary cultures in 20% O_2. However, the influence of O_2 tension on HO-1 function in healthy and OA chondrocytes remains unknown.

Methods: Human chondrocytes were obtained from healthy donors and patients with diagnosis of advanced OA undergoing total knee joint replacement. The chondrocytes were isolated by digestion with collagenase and used in primary culture. Healthy and OA chondrocyes were cultured in 20% or 1% O_2 tension, and in 5% CO_2. Cells were stimulated with IL-1β (10 ng/ml) for 48h. HO-1 was induced by incubation with 10 μM cobalt protoporphyrin IX (CoPP). Protein expression was assessed by Western blot, ELISA and immunocytochemistry. Nitrite production and matrix metalloproteinase (MMP) activity were evaluated by fluorometric methods. HO-1 gene silencing was achieved by using a gene-specific siRNA.

Results: HO-1 protein was expressed in both healthy and OA chondrocytes in hypoxia (1% O_2) and normoxia (20% O_2). IL-1β down-regulated HO-1 expression in all conditions whereas CoPP treatment counteracted this effect. CoPP treatment was able to reduce the levels of TNFα and MMP activity after IL-1β stimulation. CoPP also decreased the production of nitrite induced by IL-1β in healthy and OA cells at both O_2 concentrations. This effect was accompanied by a reduction in iNOS expression at 24h. In hypoxic conditions HIF-2α and SOX9 expression was decreased by IL-1β in both healthy and OA chondrocytes. However, HO-1 induction was able to reverse this effect and prevented the decrease in type collagen II. In addition, IL-1β induced HIF-1α expression irrespective of O_2 tension whereas HO-1 induction by CoPP down-regulated HIF-1α expression in OA chondrocytes only.

Conclusions: HO-1 induction in primary chondrocytes cultured in hypoxic conditions resulted in stronger anti-inflammatory and chondroprotective effects compared with normoxia. These results suggest that HO-1 could be a physiologically important chondroprotective factor.

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HUMAN OSTEOARTHRITIS SYNOVIUM CONTAINS AN ALTERNATIVELY SPLICED TRANSCRIPT OF ADAMTS4

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Purpose: The characterization of an alternatively spliced transcript of the ADAMTS4 aggrecanase.

Methods: In human OA synovial cell cultures, RT-PCR was performed using oligonucleotide primers designed to amplify across the exon 8/9 region of human ADAMTS4. The PCR products were purified using a QIAquick purification kit (Qiagen) and sequenced using in house facilities. A pCEP4 (Invitrogen) mammalian expression vector containing ADAMTS4 plus a FLAG epitope was mutated using the QuikChange II site directed mutagenesis kit (Stratagene) to contain the ADAMTS4 splice variant plus a FLAG epitope. The recombinant proteins were purified from HEK293 transfected cells using Anti-FLAG M2 affinity gel (Sigma). Polyclonal antibodies were raised against synthetic peptides representing sequences within the C-terminal region of the splice variant of ADAMTS4 and the raised antibodies were characterized using the recombinant splice variant of ADAMTS4. The antibodies were used in immunohistochemical analysis of human osteoarthritic synovium. The proteolysis of aggrecan and other proteoglycans by the recombinant spice variant of ADAMTS4 was investigated.

Results: The degradation of aggrecan is mainly mediated by the aggrecanases, of which ADAMTS4 (aggrecanase-1) and ADAMTS5 (aggrecanase-2) are the best known. We here characterize an alternative splice variant of ADAMTS4.

RT-PCR performed as described above resulted in the amplification of normal ADAMTS4, and also a smaller product missing 161 base pairs from the 5' end of exon 9, the result of alternative splicing in which exon 8 joins to a cryptic 3' splice site within exon 9. The protein produced by this alternative splicing would lack the spacer domain and have a C-terminus lacking any homologies with the normal ADAMTS4 spacer domain. The alternatively spliced transcript of ADAMTS4 was found in cultured OA synovial cells and in freshly digested OA synovium, but not in human brain, cervix or lung, or in normal bovine synovium. The protein synthesized from this alternatively spliced transcript of ADAMTS4 would lose functions dependent on its spacer domain, like substrate and matrix binding, and inhibition through fibronectin. Removal of the spacer domain from ADAMTS4 has been reported to increase its ability to cleave aggrecan at the Glu373-Ala374 bond, and it may well be that the alternatively spliced transcript produces a protein that is secreted in a more active form.

HEK293 cells transfected with a pCEP4 vector containing the cDNA sequence of the splice variant of ADAMTS4 produced the corresponding protein in both the pro and active form. This protein could be found in the media, but mostly associated with the cells, as confirmed using antibodies specific for the splice variant that were produced using synthetic peptides. Immunohistochemical analysis of osteoarthritic synovium using these antibodies showed staining of cells within the synovium. Proteins purified by immunoprecipitation by Anti-FLAG M2 affinity gel from transfected and untransfected HEK293 cells were analysed using the ANASpec SensoLyte 520 Aggrecanase I assay kit. The splice variant had aggrecanase activity comparable to a commercially available ADAMTS4. The splice variant cleaved aggrecan at the G1u373-A1a374 site, as assessed by the neoepitope monoclonal antibody BC3, with activity comparable to ADAMTS4.

Conclusions: ADAMTS4 is regulated at multiple levels through control of gene expression, mRNA splicing and protein processing, as well as the expression of naturally occurring inhibitors. We here describe the characteristics of the first known splice variant of ADAMTS4. This alternative splice transcript of ADAMTS4 is expressed as a protein in vivo and can be found in the synovium. It can be speculated that the changes in the C-terminal domain of the protein resulting from this alternatively spliced transcript would have changes in its substrate specificity. The protein produced by the alternative spliced transcript of ADAMTS4 has aggrecanase activity, and the release of low levels of this fully active variant of ADAMTS4 might be a factor in the slow process of superficial zone aggrecan loss in osteoarthritis.

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ANGIOPOIETIN-LIKE 4 PROMOTES TERMINAL CHONDROGENIC DIFFERENTIATION

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Purpose: Mesenchymal stem cells (MSCs) are an attractive cell source for cartilage tissue engineering given their ability to differentiate into